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<p>(54) Title: 5-LIPOXYGENASE GENE POLYMORPHISMS AND THEIR USE IN CLASSIFYING PATIENTS</p> <p>(57) Abstract</p> <p>The present invention provides identification of sequence polymorphisms in the 5-lipoxygenase gene that are present in general and asthmatic populations. The invention provides methods associated with correlating such polymorphisms with asthmatic phenotype and responsiveness to therapy. In particular, the invention demonstrates that individuals with reduced 5-lipoxygenase expression (or activity) are less responsive to treatment with 5-lipoxygenase inhibitors.</p>			

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5-LIPOXYGENASE GENE POLYMORPHISMS AND THEIR USE IN  
CLASSIFYING PATIENTS

**Related Applications**

5        The present application is a Continuation-in-part of U.S. Serial Number 60/016,890, filed May 6, 1996, the entire contents of which are incorporated herein by reference.

**Government Support**

10       Development of the present invention was supported, in part, by National Institutes of Health grant number HL-19170. The United States Government may have certain rights in the invention.

**Background of the Invention**

15       Leukotrienes are potent pro-inflammatory mediators that are synthesized by certain bone-marrow derived granulocytes after activation (Samuelsson, *Science* 220:568, 1983; Lewis et al., *J. Clin. Invest.* 73:889, 1984). Leukotrienes have been postulated to play an important role in bronchial asthma (Margoske, *Ann. NY Acad. Sci.* 629:148, 1990) and in other inflammatory diseases, such as 20       ulcerative colitis, bronchitis, sinusitis, psoriasis, allergic and non-allergic rhinitis, lupus, and rheumatoid arthritis. 5-lipoxygenase is the first committed enzyme in the pathway leading to leukotriene synthesis and is responsible for the conversion of arachidonic acid to LTA<sub>4</sub> via the unstable intermediate 5-hydroperoxy

eicosotetraenoic acid (Samuelsson et al., *Science* 237:1171, 1987; Samuelsson, *Science* 220:568, 1983).

It has been established that treating patients with agents that have the capacity to inhibit 5-lipoxygenase results in improvement in lung function, 5 reduction in asthma symptoms, and decreased need for alternative asthma treatments (Persson et al., *Anesthesiology* 82:969, 1995; Israel et al., *Ann. Int. Med.* 119:1059, 1993). Treatment with 5-lipoxygenase inhibitors can also benefit ulcerative colitis patients (Laursen et al., *Lancet* 335:683, 1990) and those suffering from rheumatoid arthritis (Weinblatt et al. *J. Rheumatol.* 19:1537, 10 1992). However, at least in the context of asthma, not all patients experience beneficial effects; there is a heterogenous response in the patient population to treatment with 5-lipoxygenase inhibitors. Prior to the present invention, the reason for this heterogeneity was not known. Moreover, there is currently no way to identify in advance those patients who will respond well, and those who will 15 respond poorly, to 5-lipoxygenase inhibitor treatment. Accordingly, there is a need for improvements in the area of patient response. There is also a need for further analysis of 5-lipoxygenase gene regulation.

#### **Summary of the Invention**

20 The present invention encompasses the discovery that polymorphisms in 5-lipoxygenase gene exist within the general population and, in particular, within the asthmatic population. According to the present invention, such polymorphisms can be correlated with asthmatic phenotype (i.e., susceptibility to asthma and/or

severity of disease) and/or with responsiveness to asthma therapies. The present invention therefore provides methods of asthma diagnosis and therapy. Furthermore, the teachings of this invention can readily be generalized to other inflammatory diseases that involve 5-lipoxygenase (e.g., ulcerative colitis, 5 bronchitis, sinusitis, psoriasis, allergic and non-allergic rhinitis, lupus, rheumatoid arthritis, etc.).

Description and characterization of certain of the 5-lipoxygenase gene polymorphisms described herein reveal that different individuals have different capabilities for 5-lipoxygenase expression. Furthermore, differences in 5-lipoxygenase gene expression account for different degrees of patient responsiveness to 5-lipoxygenase inhibitors. Specifically, the present invention demonstrates that those patients who express reduced levels of 5-lipoxygenase, as compared with patients with normal 5-lipoxygenase gene expression (which patients are sometimes referred to as "normal controls"), show less responsiveness to treatment with 5-lipoxygenase inhibitors; conversely, those patients who express increased levels are more responsive to such treatment. Similar correlations can be expected for other agents that interfere with the leukotriene metabolic pathway (e.g., leukotriene receptor antagonists, 5-lipoxygenase activating protein inhibitors, etc.).

20 Accordingly, preferred embodiments of the present invention include methods of identifying patients who are likely to be more (or less) responsive to treatment with 5-lipoxygenase inhibitors, as compared to normal controls which methods involve identifying those patients with increased (or decreased) capability

for 5-lipoxygenase expression. Analogously, the present invention provides methods defining treatments most likely to be effective for patients with altered 5-lipoxygenase gene expression and/or activity. These findings are applicable to all inflammatory diseases in which treatment with 5-lipoxygenase inhibitors (or related agents) is appropriate and, in particular, are applicable to asthma.

5 The present invention provides a method of classifying patients suffering from an inflammatory disease which method comprises steps of i) identifying in DNA from at least one patient a sequence polymorphism, as compared with the normal 5-lipoxygenase gene (SEQ ID NO.:1), in a 5-lipoxygenase gene regulatory sequence; and ii) classifying the patient based on the identified polymorphism.

10 The invention also provides a method of identifying an asthma patient who is a candidate for treatment with 5-lipoxygenase inhibitors, which method comprises steps of i) identifying a normal control individual who does not have asthma; and ii)

15 identifying an asthma patient who expresses 5-lipoxygenase at a level higher than that at which the normal control expresses 5-lipoxygenase, which asthma patient is therefore identified as a candidate for treatment with 5-lipoxygenase inhibitors.

20 The invention further provides a method of identifying an asthma patient who is not a candidate for treatment with 5-lipoxygenase inhibitors, which method comprising steps of i) identifying a normal control individual who has normal levels of 5-lipoxygenase gene expression; and ii) identifying an asthma patient who expresses 5-lipoxygenase at a level lower than that at which the normal control

expresses 5-lipoxygenase, which asthma patient is therefore identified as not being a candidate for treatment with 5-lipoxygenase inhibitors.

Additionally, the invention provides a method of identifying an individual who is susceptible to an inflammatory disorder, which method comprises steps of 5 i) providing a nucleic acid sample from an individual; and ii) detecting in the nucleic acid sample a 5-lipoxygenase gene polymorphism that correlates with the inflammatory disorder with a P value less than or equal to 0.05, existence of the polymorphism being indicative of susceptibility to the inflammatory disorder.

The invention also provides a method of classifying an asthma patient as 10 suffering from asthma, which method comprises steps of i) providing a nucleic acid sample from an asthmatic individual; and ii) detecting in the nucleic acid sample a 5-lipoxygenase gene polymorphism that correlates with asthma with a P value less than or equal to 0.05, existence of the polymorphism being indicative of asthma.

15 The invention also provides a method of treating inflammatory disorder patients which method comprises i) identifying in genetic material of one or more inflammatory disorder patients a 5-lipoxygenase gene polymorphism that correlates with increased responsiveness to a therapeutic as compared with responsiveness of an individual lacking the polymorphism; and ii) administering the therapeutic to 20 the patient.

### Description of the Drawings

Figure 1 is a single-stranded conformational polymorphism ("SSCP") analysis of DNA from patients with asthma. Two types of polymorphisms are noted and are indicated by arrows. The DNA from the patient marked with the double arrow was sequenced, and the sequence data are presented in Figure 2.

Figure 2 presents direct cycle sequencing data of the 5-lipoxygenase gene promoter region from the patient marked with the double arrow in Figure 1. The column to the right gives the sequence read from the autoradiograph, while the box to the far right indicates the area of missing sequence.

Figure 3 is a simple sequence length polymorphism ("SSCP") analysis of DNA from patients with asthma, and shows the mobility of fragments with the wild type sequence, with the 6 base pair addition, with the 6 base pair deletion, and with the 12 base pair deletion.

Figure 4 presents automated sequencing analysis of DNA from a normal individual and from two asthmatic patients with polymorphisms in 5-lipoxygenase gene regulatory sequences.

Figure 5 is a schematic giving the location of polymorphisms identified in the transcription factor binding region of the 5-lipoxygenase gene. Numbering applies only to the normal sequence and is registered such that the "A" of the ATG start codon is +1.

Figure 6 shows the relative CAT activity, corrected for transfection efficiency, of reporter constructs in which either wild-type or polymorphism-

bearing 5-lipoxygenase sequences are linked to the *CAT* gene. Results are the mean of five experiments, each of which was performed in triplicate.

Figure 7 shows electrophoretic mobility shift assay ("EMSA") and supershift analysis of nuclear proteins from human umbilical vein endothelial cells ("HUVEC's") HUVECs with radiolabeled oligonucleotides. Panel A shows shifts on a wild type oligonucleotide; Panel B shows shifts on mutant oligonucleotides.

Figure 8 shows EMSA using recombinant Egr-1 (zinc finger region) in increasing concentrations (0, 1:500, 1:200, 1:50, and 1:10).

## 10 Description of the Sequences

SEQ ID NO.:1 is the genomic 5-lipoxygenase sequence from *homo sapiens* (GenBank accession number M 38191). SEQ ID NO.:1 includes the 5-lipoxygenase promoter and exon 1 sequence.

## 15 Description of Preferred Embodiments

### *Identification of 5-Lipoxygenase Gene Polymorphisms and Mutations*

The 5-lipoxygenase gene has been cloned, both as a cDNA (Matsumoto et al., *Proc. Natl. Acad. Sci. USA* 85:3406, 1988; Dixon et al., *Proc. Natl. Acad. Sci. USA* 85:416, 1988; Balcarek et al., *J. Biol. Chem.* 263:13937, 1988) and as a genomic clone (Hoshiko et al., *Proc. Natl. Acad. Sci. USA* 87:9073, 1990; Funk et al., *Proc. Natl. Acad. Sci. USA* 86:2587, 1989). The 5-lipoxygenase gene is approximately 85 kilobases in size and contains 14 exons and 15 introns. The region 88 to 212 base pairs upstream of the 5-lipoxygenase translation start site

contains a number of sequences known to be recognition sites for transcriptional regulators (Hoshiko et al., *Proc. Natl. Acad. Sci. USA* 87:9073, 1990, incorporated herein by reference). For example, binding sites for AP1 and Sp1, each of which can act as either a transcriptional activator or a transcriptional 5 repressor, depending on context, are found in this region. With respect to Sp1, there are 5 tandem binding sites (GGGCGG) found -147 to -176 base pairs upstream of the ATG translation start site. Deletion of these Sp1 sites reduces transcription from this promoter (Hoshiko et al., *Proc. Natl. Acad. Sci. USA* 87:9073, 1990). Sp1 sites are similarly found upstream of a variety of other genes 10 (Li et al. *Gene* 164:229, 1995; Wariishi et al., *Biochem. Biophys. Res. Commun.* 216:729, 1995; Tang et al. *Biochem. Biophys. Res. Commun.* 213:673, 1995; Khachigian et al. *J. Biol. Chem.*, 270:27679, 1995). Many of these genes have 2- 3 Sp1 binding motifs; only the 5-lipoxygenase gene has five.

As described in Example 1, we have identified 5-lipoxygenase gene 15 polymorphisms in asthmatic and non-asthmatic populations. As used herein, the term "polymorphism" means any sequence difference as compared with SEQ ID NO:1. Of particular interest are 5-lipoxygenase gene polymorphisms that represent gene mutations. As used herein, the term "mutation" means a polymorphism that has an effect on 5-lipoxygenase expression or activity. That is, 20 a "mutant" 5-lipoxygenase gene is one that is not expressed at the same level as the gene of SEQ ID NO:1 in a particular cell, and/or that encodes a product (RNA or protein) that does not have the same activity as the corresponding product of the gene of SEQ ID NO:1.

For example, a mutant 5-lipoxygenase gene might encode an RNA that is not spliced or modified in the same way as the RNA encoded by SEQ ID NO:1. Alternatively or additionally, a mutant 5-lipoxygenase gene might encode a mutant 5-lipoxygenase protein that does not behave identically to the protein encoded by

5 SEQ ID NO:1 in activity assays such as those described herein. Other examples of "mutant" 5-lipoxygenase genes will be readily recognized by those of ordinary skill in the art. Any 5-lipoxygenase gene that is expressed at a level that differs by at least about 20-30% from that of the wild-type 5-lipoxygenase gene (SEQ ID NO:1) under given expression conditions (i.e., in the same cell type, etc.) is

10 considered to be a "mutant" gene according to the present invention. Also, any gene that encodes a protein whose activity level differs by at least about 20-30% from that of the wild-type 5-lipoxygenase protein (encoded by SEQ ID NO:1) is a mutant gene. Mutant gene expression (or activity) may be increased or decreased as compared with wild-type.

15 As described in Example 1, we have identified a variety of polymorphisms in the 5-lipoxygenase gene. Specifically, using single-stranded conformational polymorphism (SSCP), simple sequence length polymorphism (SSLP), and DNA sequencing techniques, we found polymorphisms in both the coding region and the regulatory sequences of the 5-lipoxygenase gene. Coding-region polymorphisms in

20 the 5-lipoxygenase gene include substitutions in exon 1 (C→T at base 21), exon 2 (G→A at base 270) and exon 13 (A→G at base 1,728); regulatory sequence polymorphisms include G→A substitutions (at position -1,708 and -1,761 of the wild-type gene), a 12-base-pair deletion (of sequences corresponding to -165 to -

176 of the wild-type gene), a 6-base-pair deletion (of sequences corresponding to -171 and -176 of the wild-type gene), and a 6-base-pair addition (of an Sp1 site) upstream of the gene.

Those of ordinary skill in the art will recognize that the particular methods of polymorphism identification described in Example 1 are not intended to be limiting of the present invention. Any of a variety of other known techniques, such as, for example, RNA-DNA hybrid cleavage using RNase A, mismatch detection using heteroduplex analysis, denaturing gradient gel electrophoresis, and chemical cleavage of heteroduplex DNA (see, for example, *Current Protocols in Human Genetics*, Volume 1, Chapter 7, John Wiley and Sons, 1995, incorporated herein by reference) could alternatively be used. Furthermore, these techniques can be applied to other populations, for example, to populations of patients suffering from non-asthmatic inflammatory diseases (e.g., ulcerative colitis, bronchitis, sinusitis, psoriasis, allergic and non-allergic rhinitis, lupus, or rheumatoid arthritis).

5-lipoxygenase gene polymorphisms identified as described herein are classified as "mutations" or "non-mutations" by expression and/or activity assays. Any of a variety of techniques may be employed. For example, expression of polymorphism-containing 5-lipoxygenase genes may be assayed at the RNA level using techniques, such as Northern blots, Southern blots, Differential Display, *in situ* hybridization, RNase protection assays, etc. (see, for example, *Current Protocols in Molecular Biology, supra*).

Alternatively or additionally, 5-lipoxygenase regulatory sequences containing polymorphisms can be analyzed, for example, by linkage to reporter genes whose activity can be measured at the RNA or DNA level, or by binding assays that test their ability to interact with relevant protein regulatory factors.

5 Polymorphisms that contain additions, deletions, or disruptions of regulatory factor binding sites are likely to constitute 5-lipoxygenase gene mutations. A "disruption" of a regulatory factor binding site is any sequence alteration that reduces or enhances the extent to which the site is bound by the regulatory factor.

As discussed above, the 5-lipoxygenase gene contains five *Spl* sites that are 10 involved in regulating its expression. Additional regulatory sequences can readily be identified and analyzed by promoter fusion and "promoter bashing" experiments (Orkin, *J. Biol. Chem.* 10:4955, 1995; Lederer et al., *J. Immunol.* 152:77, 1994). It is important to note that, given the propensity of eukaryotic transcriptional regulators to function at a distance (Semenza, *Hum. Mutat.* 3:180, 1994; Hill et 15 al., *Cell* 80:199, 1995), "promoter" fragments for use in promoter fusion and "bashing" experiments should be chosen from upstream, downstream, within, and without the 5-lipoxygenase gene. At any rate, once regulatory sequences are identified in the wild-type gene, polymorphisms that disrupt the sequences can be classified as mutations.

20 Because binding sites for many transcriptional activators and repressors are known (Boulikas, *Crit. Rev. Eukaryot. Gene Expr.*, 4:117, 1994; Pennypacko, *Pharmacology* 51:1, 1995; Struhl, *Trends Bioch. Sci.* 14:137, 1989), those of ordinary skill in the art can readily recognize when a sequence alteration produces

a new transcriptional regulator binding site. Of course, such an alteration is only likely to affect 5-lipoxygenase gene expression in the patient (and therefore to constitute a true "mutation") if the relevant transcriptional regulator is expressed in appropriate tissues (i.e., in cells of hemopoietic origin, such as mast cells, 5 eosinophils, alveolar macrophages and related inflammatory cells). Transcriptional regulators known to be expressed in these tissues include AP1, AP3, c-fos, CK-1, CK-2, c-myc, Egr-1, GATA, GC box, NF-AT, NF $\kappa$ B, and TATA (see, for example, Cousins et al., *Am J. Respir. Crit. Care Med.* 150:S50, 1994; Priesch et al., *J. Immunol.* 155:4963, 1995). Thus, 5-lipoxygenase regulatory sequence 10 polymorphisms involving additions, deletions, or disruptions of binding sites for these factors are likely to be mutations. This fact can be confirmed by expression and/or activity studies as discussed herein.

In addition (or as an alternative) to the expression techniques discussed above, 5-lipoxygenase gene mutations may be classified through analyses of the 15 proteins they encode. For example, protein expression can be studied using immunological techniques such as Western blots, radioimmunoassays (RIA's), enzyme-linked immunoassays (ELISA's), immuno-histo-chemistry, etc. (see, for example, *Current Protocols in Molecular Biology*, Volume I and II, Ausubel et al., John Wiley and Sons, New York, 1987-96, incorporated herein by reference). 20 Alternatively or additionally, protein activity can be tested by measuring leukotriene levels or detecting 5-lipoxygenase products (see, for example, Murphy, *J. Mass Spectrometry* 30:5, 1995; Lee et al., *Ann. Rev. Resp. Dis.* 141:1453, 1990, each of which is incorporated herein by reference).

As described in Example 1, we have identified 5-lipoxygenase gene mutations by fusing polymorphism-containing regulatory regions to a *CAT* reporter gene. We also tested the ability of these polymorphism-containing regulatory sequences to interact with certain protein regulatory factors. As shown, CAT 5 activity was reduced at least about 20%, and typically at least about 50%, for each of the 6-basepair deletion, the 12-basepair deletion, and the 6-basepair insertion, as compared with a control reporter fused to the wild-type promoter (see Figure 6). Thus, each of these polymorphisms constitutes a 5-lipoxygenase gene mutation according to the present invention.

10

*Correlation of 5-Lipoxygenase Gene Polymorphisms and Mutations with Asthmatic Phenotype*

As described above, the present invention provides the discovery that 5-lipoxygenase gene polymorphisms and mutations exist within the general 15 population. The invention further teaches that such polymorphisms and mutations can be correlated with asthmatic phenotype. Specifically, according to the present invention, polymorphisms and mutations that are found more commonly among asthmatics than non-asthmatics are diagnostic of asthmatic phenotype. Preferred diagnostic polymorphisms or mutations occur a greater proportion of the time in 20 affected individuals ( $P \leq 0.05$ ). Any available technique can be used to assay the 5-lipoxygenase genotype of individuals within the population (see, for example, In et al., *J. Cell Immunol.*, 1997), incorporated herein by reference). Preferred diagnostic methods of the present invention include identifying such a correlated

mutation or polymorphism in the genome of an individual and, on the basis of that identification, classifying the individual as susceptible to or suffering from asthma. As used herein, an individual is "susceptible" to asthma if that person is statistically more likely to develop asthma than is a member of the general 5 population.

In particularly preferred embodiments of the present invention, certain 5-lipoxygenase gene polymorphisms or mutations are correlated with severity and/or nature of asthmatic phenotype (e.g., with "mild", "moderate", or "severe" asthma, as defined by established clinical parameters [see, for example, Moffitt et 10 al., *Am. Fam. Phys.*, 50:1039, 1053, 1994]; "nocturnal" or "non-nocturnal" asthma [see, for example, Busse, *Am. J. Med.*, 85:24, 1988]; etc.). For example, correlation studies as described herein are performed to identify those 5-lipoxygenase gene polymorphisms and mutations that are associated with more or less severe forms of asthma. Once identified, such polymorphisms or mutations 15 may then be used to diagnose patients suffering from the relevant form of asthma.

*Correlation of 5-Lipoxygenase Gene Polymorphisms and Mutations with Response to Therapy*

In particularly preferred embodiments of the present invention, 5-lipoxygenase gene polymorphisms or mutations are correlated with patient 20 responsiveness to particular pharmacologic therapy. According to the present invention, 5-lipoxygenase gene mutations that decrease 5-lipoxygenase gene expression or activity are likely to correlate with reduced patient responsiveness to

treatment with agents, such as 5-lipoxygenase inhibitors, that perform their therapeutic function by antagonizing 5-lipoxygenase. Conversely, mutations that increase 5-lipoxygenase expression are likely to correlate with increased responsiveness to such therapy. As will be understood by those in the art, agents 5 "antagonize" 5-lipoxygenase if they reduce or interfere with its activity or expression.

The present invention demonstrates that alterations in 5-lipoxygenase gene regulatory sequences can disrupt gene expression. Insertion of new binding sites for transcriptional activators, deletion of binding sites for transcriptional 10 repressors, or sequence substitutions that disrupt repressor binding sites or create binding sites for known activators, are likely to increase 5-lipoxygenase gene expression; conversely, sequence alterations that delete or destroy activator sites, or introduce repressor sites, are likely to decrease 5-lipoxygenase expression. In 15 particular, deletion or addition of Sp1 and/or Egr-1 sites decreases expression at least about 20% as compared with wild-type (see Example 1). Furthermore, each of these mutations correlates with reduced responsiveness to ZYFLO™ zileuton (available from Abbott Laboratories, Abbott Park, IL), a 5-lipoxygenase inhibitor 20 (see Example 2).

5-lipoxygenase gene mutations that decrease (or increase) gene expression 20 or activity are identified as described herein. Any available technique may then be used to correlate such mutations with responsiveness to therapy. For example, as described in Example 2, a broad trial analyzing therapy effectiveness may be conducted and the data may subsequently be stratified according to 5-lipoxygenase

genotype. Alternatively or additionally, the patient and control populations may first be segregated by 5-lipoxygenase genotype and then therapy effectiveness trials may be performed on each group. Any known asthma therapy may be tested in this way. For example,  $\beta$ -agonists, such as albuterol, may be so tested.

5 Therapies administered for other inflammatory diseases (e.g., ulcerative colitis, bronchitis, sinusitis, psoriasis, allergic and non-allergic, rhinitis, lupus, rheumatoid arthritis, etc.) may also be so tested. New inflammatory disease therapies may also be evaluated in this way.

The present invention therefore provides methods of identifying therapies  
10 whose effectiveness varies with 5-lipoxygenase genotype, and therefore provides methods of identifying patients who are more (or less) likely to respond to a particular therapy by detecting the 5-lipoxygenase genotype in those patients. In particular, the invention provides methods of identifying patients who are less likely to be responsive to therapies whose pharmacologic action involves inhibiting  
15 or disrupting 5-lipoxygenase activity, which methods involve detecting those patients who carry 5-lipoxygenase gene mutations that reduce the gene's expression or activity level. Such patients would also presumably be more responsive to agents that increase 5-lipoxygenase activity or expression. Furthermore, such patients would also be less likely to be responsive to treatments  
20 that interfere with other aspects of the leukotriene metabolic pathway (such as, for example, leukotriene receptor antagonists, 5-lipoxygenase activating protein inhibitors, etc.).

The present invention may be illustrated by the use of the following non-limiting Examples.

### Examples

5   **EXAMPLE 1: Isolation of 5-Lipoxygenase Gene Polymorphisms**

*Materials and Methods*

PATIENT MATERIALS: Fresh whole blood was obtained from 25 patients (20 males) with asthma, according to American Thoracic Society (ATS) criteria, whose only asthma treatment was inhaled  $\beta$ -agonists. The forced expiratory volume in the first second ("FEV"), in these patients averaged 62.3% of predicted levels and their mean age was 39.1 yr. Fresh whole blood was also obtained from 6 patients with aspirin-sensitive asthma whose clinical characteristics had been previously detailed (Israel et al., *Am. Rev. Respir. Dis.* 148:1447, 1993) and 25 individuals (11 males) who had no history of significant medical illness, had no history of asthma or atopy, and had negative skin tests to a panel of 12 common aeroallergens. Buffy coat was separated, resuspended in RPMI, layered onto a Percoll gradient, and immortalized using Epstein-Barr virus by standard techniques (Sugden et al., *J. Virol.* 23:503, 1977; Neitzel et al., *Hum. Genet.* 73:320, 1986).

DNA was obtained by buccal brushing from an additional 81 patients with 20 mild to severe asthma (none with aspirin-sensitive asthma by history), who received their asthma care at the Longwood Medical Area Adult Asthma Center (Boston, MA); these samples were used to confirm the presence of the mutations noted in the genomic DNA isolated from the immortalized cell lines noted above.

In these patients the diagnosis of asthma, according to American Thoracic Society criteria, was confirmed by chart review.

**POLYMORPHISM IDENTIFICATION:** Genomic DNA within the 14 exons, the known positive regulatory region (854 to 931 bp upstream from the ATG start site), and negative regulatory regions (292 to 727 and 1,557 to 1,884 bp upstream from the ATG start site) was screened for polymorphisms using single-stranded conformational polymorphism (SSCP) analysis according to the method of Orita et al. (*Geronics* 5:874, 1989), with minor modifications; the oligonucleotide primers used are listed in Table I. The kinase labeling reaction was carried out by mixing 3.0  $\mu$ l of [ $\gamma$ <sup>32</sup>P] ATP (3,000 Ci/mmol, 10  $\mu$ Ci/ $\mu$ l), 1.7  $\mu$ l of 20- $\mu$ M primers each, 37.1  $\mu$ l dH<sub>2</sub>O, 5.0  $\mu$ l of 10 X T4 polynucleotide kinase buffer, and 1.5  $\mu$ l of T4 polynucleotide kinase (10 U/ $\mu$ l). The mixture was incubated at 37 °C for 45 min and then at 66-70 °C for 10 min. The PCR reaction volume was 12.5  $\mu$ l containing 100-250 ng of genomic DNA in the presence of 1  $\mu$ l of <sup>32</sup>P-labeled primers (0.13  $\mu$ M each), dNTPs (0.4 mM each), MgCl<sub>2</sub> (1.6 mM) and Taq DNA polymerase (0.5 U).

TABLE I  
PCR Primers Used in Mutation Identification and Sequencing of the 5-Lipoxygenase Gene

Region of Interest	Sense primer (5' to 3')	Antisense primer (5' to 3')
Negative regulatory region* (-1,557 to -1,844)	AAAGAACAGCGTTGGTGGAT	CAAATTCAATTGTGTTGCATGTG
(-292 to -727)	ACCACTGGCCATCTCTGG	CTCTCAGCGAGGTGTCTG
Positive regulatory region* (-854 to -931)	TTAGCCGAGATCAATAACACGC	GGGTGTGGAGAAGGTTTCG

	Transcription factor binding region* (-88 to -212)	AGGAACAGACACCTCCTGAGGAGCA	GAGCAGCGAGCGGGAGCCTCGGC
5	Exon 1	CGCCATGCCCTCCTACAC	CCACGCTGGAAGTCGTTGTA
	Exon 2	TCTCCTTCCCAGGTGGAT	GCTCACCGCGTCC
	Exon 3	CTTTCTTACAGCAAAGTTG	taxaACTCACCGATATTG
	Exon 4	TCTCATGCTCAGATGGATG	ACTTACGCTTGGA
	Exon 5	TGGAGAACCTGTTCATCAACC	TCACCAAGAAATAGTGTGCTGA
10	Exon 6	CCTGGTAGAGCGGGTCATGAATC	ACCTCCTGCTCCAAGCTGAGCT
	Exon 7	TCGCTCCTGCAGCAAGGG	GCCTACCTGGAT
	Exon 8	CCAATGTATGAGCTCAAC	getataCTGTACCTTGAA
	Exon 9	CCCTCCCCAGCTGCTGGT	ACCCACCTTGTCAAAGCGGCC
	Exon 10	CACTCCAGGCCAACGCCACA	CCACACCAGGAGCCCCTCGT
	Exon 11	CCCCTGAGCCAGGTTACG	ccacgaaCCCTACCTGAGGA
15	Exon 12	TTCCACCCCTAGGCTTCCC	GCCTACCTGGCCGAAGTT
	Exon 13	GTGCTCCTGGATCCCCAAT	CTCGTTTCTGGAACTGGC
	Exon 14	CTGGGCCCTCAGCTGTTG	CACATGGAACAAGACCCATT

\*Position is reference to the ATG translation start site as +1.

20                   Amplification of the highly G+C-rich transcription factor binding region  
 (88 to 212 bp upstream from the ATG start site) required different PCR  
 conditions. A reaction volume 20  $\mu$ l was used containing 250 ng of genomic  
 DNA in the presence of 2  $\mu$ l of  $^{32}$ P-labeled primers (0.2  $\mu$ M of each primer),  
 dATP (0.2 mM), dCTP (0.2 mM), dTTP (0.2 mM), a 3:1 ratio of dGTP to 7-  
 25 deaza-dGTP (0.2 mM), 1  $\mu$ l of DMSO, 2  $\mu$ l of Taq extender PCR additive  
 (Stratagene Inc., La Jolla, CA), and Taq polymerase (1 U). PCR was performed  
 (PTC-100TM programmable thermocycler; MJ Research, Inc., Waltham, MA) for

35 cycles (each cycle was 94 °C for 1 min, 56-62 °C for 1 min, and 72 °C for 1 min).

PCR products were diluted (1:10) in a sequencing stop solution (95% formamide, 20 mM ethylenediaminetetraacetic acid ("EDTA") EDTA, 5 0.05 % (w/v) xylene cyanol, 0.05 % (w/v) bromophenol blue). Denaturation was carried out at 95 °C for 10 min and samples were immediately loaded on a nondenaturing acrylamide gel (AT Biochem, Malvern, PA). Electrophoresis was performed, using a DNA sequencing apparatus, at a constant temperature of 4 °C and at 40 W. After 5 to 6 hours, the gel was dried and exposed to X-ray film.

10 SIMPLE SEQUENCE LENGTH POLYMORPHISM (SSLP): Because sequence analysis showed polymorphisms that modified the number of Sp1 and Egr-1 binding motifs, the transcription binding region (212 to 88 bp upstream from the translation start site) of genomic DNA from a number of individuals was analyzed by SSLP (Weber et al., *Am. Hum. Genet.* 44:388, 1989). SSLPs were carried out 15 either using <sup>32</sup>P-labeled primers or including [ $\alpha$ -<sup>35</sup>S] dATP in the PCR reaction mix. PCR amplification reactions for the <sup>32</sup>P-labeled primers were performed similar to the method used for SSCP. The amplification of genomic DNA that involved the inclusion of [ $\alpha$ -<sup>35</sup>S] dATP was carried out according to the following method. The PCR mixture in 25  $\mu$ l contained 500 ng of genomic DNA, PCR 20 buffer (Boehringer Mannheim, Mannheim, Germany), 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dCTP and dTTP, 50  $\mu$ M dGTP, 150  $\mu$ M 7-deaza-dGTP, 10  $\mu$ M dATP, 6.25  $\mu$ Ci of [ $\alpha$ -<sup>35</sup>S] dATP (Dupont-New England Nuclear, Boston, MA), 10 pmoles of each primer, 5% DMSO, and 1.5 U of Taq polymerase. The PCR conditions were: 6

min at 94 °C, followed by 31 cycles of 94 °C for 15 s, 62 °C for 23 s, and 72 °C for 30s. Chain elongation was continued after the last cycle for 5 min. 10  $\mu$ l of PCR product was mixed with 5  $\mu$ l of stop solution. Samples were denatured at 95 °C for 5 min, and 6  $\mu$ l of each sample was loaded on a 6% denaturing acrylamide 5 gel; the gel was run at room temperature for 2.5 h at 60 W. Gels were dried and exposed to X-ray film as required (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, CSH, NY).

DNA SEQUENCING: Direct cycle sequencing was performed on target sequences that were amplified by using the primers employed for PCR-SSCP 10 analysis. The PCR mixture contained 2  $\mu$ M of both primers, 0.2 mM of dNTP mixture, 0.5  $\mu$ g of genomic DNA, reaction buffer (1X), and 1.25 units of Taq polymerase (GIBCOBRL) in a total volume of 50  $\mu$ l. PCR was performed in a PTC-100TM programmable thermocycler for 30 cycles (each cycle was 94 °C for 30 s, 56-62 °C for 30 s, and 72 °C for 1 min). The PCR products were purified 15 by PCR product purification Kit (Promega), and were used as template DNA in direct cycle sequencing reactions.

PCR primers were used as the sequencing primers. Primers were labeled using T4 polynucleotide kinase using the following reaction mixture: 1  $\mu$ l primer (20 pmol/ $\mu$ l), 1  $\mu$ l 10 X kinase reaction buffer, 4.5  $\mu$ l [ $\gamma$ -<sup>32</sup>P]ATP (1700 Ci/mmol, 20 10 mCi/ $\mu$ l), 2.5  $\mu$ l H<sub>2</sub>O, 1  $\mu$ l T4 polynucleotide kinase (10 U/ $\mu$ l). The mixture was incubated at 37 °C for 30 min, then heated to 90 °C for 10 min to inactivate the kinase.

One microliter of labeled primer was mixed with template DNA (60-80 fmol), 4.5  $\mu$ l of reaction buffer (300 mM Tris-HCl, pH 9.0, 50 mM MgCl<sub>2</sub>, 300 mM KCl) and 1.25 units of Taq DNA polymerase (GIBCOBRL, Gaithersburg, MD) in a total volume of 36  $\mu$ l. Then, 8  $\mu$ l of the labeling mixture were 5 transferred to each of the 4 terminal tubes containing 2  $\mu$ l of the termination mixture (A, G, C and T) as described in the dsDNA Cycle Sequencing System Kit (GIBCOBRL). PCR amplification was carried out with 20 cycles of a denaturation step at 94 °C for 30 s, an annealing step at 56 °C for 30 s, and an extension/termination step at 72 °C for 1 min, followed by 10 cycles of a 10 denaturation step at 94 °C for 30 s and an extension/termination step at 72 °C for 1 min. The reaction was stopped with 5 ml stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). All samples were incubated at 90 °C for 2 min and 3  $\mu$ l of each sample were loaded onto an 6% sequencing gel. To sequence both alleles from heterozygous individuals, DNA 15 from two clones (wild type and mutant) from TA cloning that showed migration differences on a 3% agarose gel were sequenced.

Automated sequencing was performed at Children's Hospital (Boston) Sequencing facility using ABI (Applied Biosystems, Inc., Foster City, CA) technology.

20 **CHLORAMPHENICOL ACETYL TRANSFERASE (CAT) REPORTER ASSAYS:** Plasmids 5LO-CAT were constructed using the pCRII vector (Invitrogen Corp., San Diego, CA) or the pCAT-Enhancer vector (Promega Corp., Madison, WI) as indicated by each respective manufacturer (see In et al., *J. Clin. Invest.*, 99:5, 1997,

incorporated herein by reference). PCR-amplified segments from genomic DNA isolated from individuals who harbored the various polymorphisms identified in the 5-LO gene promoter were subcloned into the pCRII (p5LOpCRII) vector. (In wild type DNA, the amplified segment was 280 bp and consisted of the 5-LO DNA 294 to 15 bp upstream from the ATG translation start site. The size of the amplified fragment differed among the various polymorphic forms of DNA.) The 5-LO upstream region was isolated from p5LOpCRII by restriction enzyme digestion with *Hind*III and *Xba*I and ligated into the pCAT-Enhancer vector to generate p5LO-CAT. HeLa cells were cultured in 60-mm diam culture dishes in modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum ("FCS"). p5LO-CAT was transfected into these cells when they reached 50-70% confluence using Transfectam (Promega Corp.). Cells were harvested 48 h after transfection, lysed and lysates analyzed for CAT activity by standard techniques (Finn et al., *Proc. Natl. Acad. Sci. USA* 87:914, 1990; Ausubel et al., *Current Protocols in Molecular Biology* John Wiley & Sons, NY, 1993). The transfections were internally controlled by cotransfection with the plasmid vector pXGH5 containing the human growth hormone (HGH) structural sequence (Nichols Institute, San Juan Capistrano, CA). Human growth hormone activity of the cell supernatant was used to normalize the CAT results from transfection efficiency using the HGH-TGES 100T kit (Nichols Institute) according to the manufacturer's instructions.

**OLIGONUCLEOTIDE SYNTHESIS, PURIFICATION, AND RADIOLABELING:**

Oligonucleotides for supershift analysis and electrophoretic mobility shift assay (EMSA) using recombinant proteins were synthesized on a 392 DNA/RNA

Synthesizer (Applied Biosystems, Inc.). Complementary strands were annealed, purified by nondenaturing polyacrylamide gel electrophoresis, and end-radiolabeled with [ $\gamma$ -<sup>32</sup>P] ATP (DuPont-NEN, Boston, MA) using T4 polynucleotide kinase (New England BioLabs Inc., Beverly, MA).

5                   NUCLEAR EXTRACT PREPARATION: Compartmental extracts from HUVEC's grown in tissue culture were prepared by a modification of the method of Dignam et al. (*Nuc. Acids Reg.* 11:1475, 1983). At confluence, monolayers were washed twice with PBS at 4 °C and collected, using a rubber policeman, in a 50-ml conical tube on ice. Cells were pelleted and lysed, and the nuclei were pelleted; 10 the nuclei were then resuspended, lysed, vortexed, and centrifuged (Khachigian et al., *Science* 271:1427, 1996). Supernatants containing nuclear proteins were removed, immediately frozen on dry ice, and stored at -80 °C.

RECOMBINANT PROTEINS: Recombinant Egr-1 (zinc finger region) was generously provided by Dr. F.J. Raucher III (Wistar Institute of Anatomy and 15 Biology, University of Pennsylvania, Philadelphia, PA). Samples were stored at -80 °C in dilutions of 1:10, 1:50, 1:200, and 1:500 containing 25 mM Hepes-KOH, pH 7.5, 100 mM KC1, 10 mM ZnSO<sub>4</sub>, 0.1% Nonidet P-40, 1 mM DDT, and 50% glycerol. Recombinant Sp1 (Promega Corp.), purified from HeLa cells infected 20 with recombinant vaccinia virus containing human Sp1 cDNA, was stored similarly in 12 mM Hepes-KOH, pH 7.5, 50 mM KC1, 6 mM MgCl<sub>2</sub>, 5 mM ZnSO<sub>4</sub>, 0.05% Nonidet P-40, 1 mM DTT, and 50% glycerol.

#### ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) AND SUPERSHIFT

ANALYSIS: *In vitro* binding reactions between oligonucleotides and nuclear extract

were done in a total volume of 20  $\mu$ l containing: 2  $\mu$ l of 10 X binding buffer (0.1 M Tris-HCl, pH 7.5, 50% glycerol, 10 mM EDTA, 10 mM dithiothreitol), 1  $\mu$ l of 1 mg/ml poly(dI.dC) (Sigma Chemical Co.), 1  $\mu$ l of 1 mg/ml salmon sperm DNA (Sigma Chemical Co.), 3  $\mu$ l of nuclear extract (normalized to ~8 mg/ml of total protein), and 1  $\mu$ l radiolabeled oligonucleotide (specific activity 50,000 cpm/reaction). Binding reactions involving recombinant proteins contained 2  $\mu$ l of the appropriate dilution and 1  $\mu$ l of mg/ml BSA as a protein carrier. The reaction was allowed to proceed for 30 min at 22 °C before the addition of nondenaturing loading buffer (0.2% bromophenol blue, 0.2% xylene cyanol, 20% glycerol).

5 Samples were electrophoresed for ~1.5 h. Gels were dried under vacuum and autoradiographed overnight. Supershift studies were performed as above except 1  $\mu$ l (1  $\mu$ g) of the appropriate antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added 10 min before the addition of radiolabeled oligonucleotide. When appropriate, densitometric analysis was completed on the autoradiographs using

10 15 ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

STATISTICS: CAT activity relative to HGH transcription of the various constructs was compared by analysis of variants ("ANOVA"); when significant differences were noted among groups, they were compared by Student's *t* test. Differences in allele frequency among groups were calculated using Fisher's exact

20 test..

**Results**

CODING REGION POLYMORPHISMS: Polymorphisms consisting of a change in a single base pair were identified in exon 1 (C→T at base 21), exon 2 (G→A at base 270), and exon 13 (A→G at base 1,728). At the locus identified in exon 1, 5 there were 6 heterozygotes among the 25 nonaspirin-sensitive asthmatic subjects, 3 among the 6 aspirin-sensitive asthmatic subjects, and 5 among the 25 normal subjects; allele frequencies are given in Table II.

TABLE II				
Allele Frequencies at 5-LO Gene Polymorphisms				
Locus	Allele type	Allele frequency		
		Asthmatic		Normal
		Nonaspirin-sensitive	Aspirin-sensitive	
Negative regulatory region (-1557 to 1,844)	Wild-type	0.88 (44/50)	0.83 (10/12)	0.80 (40/50)
	G1708→A G1761→A	0.12 (6/50)	0.17 (2/12)	0.20 (10/50)
Transcription factor binding region	Wild type	0.78 (165/212)	0.75 (9/12)	0.76 (38/50)
	12-bp deletion*	0.06 (12/212)	0.00 (0/12)	0.02 (1/50)
	6-bp deletion*	0.14 (30/212)	0.25 (3/12)	0.18 (9/50)
	6-bp addition*	0.02 (5/212)	0.00 (0/12)	0.40 (2/50)
Exon 1	Wild type	0.88 (44/50)	0.75 (9/12)	0.90 (45/50)
	C21→T	0.12 (6/50)	0.25 (3/12)	0.10 (5/50)
Exon 2	Wild type	0.94 (47/50)	1.00 (12/12)	1.00 (50/50)

	G270→A	0.06 (3/50)	0.00 (0/12)	0.00 (0/50)
Exon 13	Wild type	0.92 (46/50)	1.00 (12/12)	0.94 (47/50)
	A1728→A	0.08 (4/50)	0.00 (0/12)	0.06 (3/50)

Allele frequencies in the nonaspirin-sensitive asthmatic subjects are given for the 25 patients with immortalized DNA and the 81 patients from whom cheek brushings were available. \*Location of the additions/deletions identified in the text.

5

At the locus identified in exon 2, there was one heterozygote and one homozygote among the 25 nonaspirin-sensitive asthmatic subjects; this polymorphism was not found in the 6 aspirin-sensitive asthmatic or the 25 normal subjects. At the locus identified in exon 13, there were 4 heterozygotes among the 10 25 nonaspirin-sensitive asthmatic subjects, no heterozygotes among the 25 nonaspirin-sensitive asthmatic subjects, no heterozygotes among the 6 aspirin-sensitive asthmatic subjects, and 1 heterozygote and 1 homozygote among the 25 normal subjects. Each of these polymorphisms represents a conservative substitution in that the amino acid sequence of the putative 5-LO protein derived from the polymorphic DNA would not be changed. In the sample of DNAs examined, there was no significant difference in the allele frequency of each of these polymorphisms between the normal and asthmatic subjects. Among the 25 normal and 31 asthmatic individuals examined, there were no polymorphisms noted by SSCP in exons 3-12 and 14.

20

**POLYMORPHISMS IN THE 5' FLANKING REGION:** Polymorphisms, consisting of a change in a single base pair, were noted in the negative regulatory region (as defined by Hoshiko et al. [Proc. Natl. Acad. Sci., USA, 86:2587, 1989] between

positions 1,844 and 1,557 upstream from the ATG translation start site; these consisted of a G→A substitution at -1,708 and a G→A substitution at -1,761. All subjects with the G→A substitution at -1,708 also had the G→A substitution at -1,761. There were six nonaspirin-sensitive asthmatics (heterozygotes), two 5 aspirin-sensitive asthmatics (heterozygotes), and nine (eight heterozygotes and one homozygote) normal subjects with these mutations.

A family of polymorphisms was noted in the transcription factor binding region 212 to 88 bp upstream from the translation start site. The first member of this family was originally termed a "type I" polymorphism by SSCP as defined in 10 Figure 1. This polymorphic allele was present in two samples (one homozygote and one heterozygote) of immortalized lymphocyte DNA from non-aspirin-sensitive asthmatic subjects and one normal subject (heterozygote); it was not identified in any aspirin-sensitive asthmatic subjects. Direct cycle sequencing of the DNA obtained from the individual homozygous for this polymorphic allele, 15 Figure 2, indicated the presence of a 12-bp deletion, -GGGCGGGGGC GG- from the wild-type sequence between positions -165 and 176. Interestingly, this deletion consisted of two of the known five tandem repeats of the consensus Sp1 binding sequence (Karin et al., *Nature* 308:513, 1984; Ishii et al., *Science* 232:1410, 1986; Harrington et al., *Proc. Natl. Acad. Sci. USA* 85:2066, 1988), which are 20 present between 147 and 176 bp upstream from the ATG start site of the 5-LO gene.

The DNA from individuals harboring the "type II" polymorphism in Figure 1 was resolved using automated sequencing as a 6-bp deletion consisting of a loss

of a single Sp1 and Egr-1 binding motif between positions -171 and -176. Because these polymorphisms, i.e., a deletion of one and two Sp1 or Egr-1 binding motifs, substantially modified the length of the DNA fragments obtained by PCR, we screened genomic DNA from an additional 81 asthmatics using SSLP. This led to 5 the identification of a third polymorphic form of DNA, Figure 3, identified by automated sequencing as a 6-bp addition of a Sp1 binding domain. The polymorphic alleles identified at this locus are compared to the wild-type sequence in Figure 5. Among the 61 individuals from whom immortalized DNA was available, there were 23 individuals harboring polymorphic alleles as indicated by 10 SSLP. In 14 of these individuals, the inferred polymorphism was confirmed by automated sequencing (data not shown).

**HAPLOTYPIC REFERENCES:** When DNA sequences from the wild type and polymorphic DNA sequences were compared, it was determined that individuals heterozygous or homozygous for the 12-bp deletion were also heterozygous or 15 homozygous for the polymorphism noted in exon 2, respectively. Similarly, we determined that individuals heterozygous or homozygous for the 6-bp deletion were also heterozygous for the polymorphism noted in exon 1. These findings are consistent with the presence of a haplotype in which the 6- and 12-bp transcription 20 factor binding region polymorphisms are in linkage disequilibrium with the previously noted exon 1 and exon 2 polymorphisms, respectively.

**CAT REPORTER ASSAYS:** To determine whether functional differences existed among the various forms of the 5-LO transcription factor binding region that we had identified, we constructed a panel of CAT reporter gene constructs containing

the wild type, the 12-bp deletion, the 6-bp deletion or the 6-bp addition. The CAT activity in relationship to the activity of the co-reporter plasmid pHGH is shown in Figure 6; data shown are the mean of 5 separate transfections, each performed in triplicate. The relative CAT activity of the constructs containing the 5 12-bp deletion, the 6-bp deletion, or the 6-bp addition was significantly less than the CAT activity of the wild type constructs,  $P=0.0047$ ,  $P=0.022$ , and  $P=0.038$ , respectively. Each of these polymorphisms therefore constitutes a 5-LO gene mutation.

EMSA: To further characterize the wild type and mutant forms of the 5-LO transcription factor binding region, EMSA's were used to detect specific DNA-nuclear protein interactions. The ubiquitous transcription factor SP1 is known to bind the functionally important G+C-rich region of the 5-LO promoter (Hoshiko et al., *Proc. Natl. Acad. Sci. USA* 87:9073, 1990). The inducible transcription factor Egr-1 shares a similar G+C-rich consensus binding motif with Sp1, and 15 Sp1/Egr-1 interactions may be important in the transcriptional regulation of many genes (Khachigian et al., *Science* 271:1427, 1966). We therefore proposed that Egr-1 would also be capable of interacting with the 5-LO G+C-rich sequence as well as Sp1. Moreover, we hypothesized that Sp1 and Egr-1 binding differences may be partially responsible for the functional differences detected with our CAT 20 reporter assays.

Because HUVEC are known to produce Sp1 and phorbol-myristate-acetate-(PMA)-inducible Egr-1, they were used as a source of nuclear extracts. EMSA with unstimulated HUVEC nuclear extract produced a single intense band (Figure

7). Nuclear extract prebound to anti-Sp1 antibodies supershifted the upper band, whereas anti-Egr-1 antibodies supershifted the lower band. Identical concentrations of antibodies to Sp1 and ETS (a nuclear binding protein with transactivating properties) factors produced no change. These data suggest that 5 Sp1 and inducible Egr-1 are capable of binding the wild type G+C-rich region of 5-LO.

EMSA's with the three mutant G+C-rich regions demonstrated that they are also capable of binding Sp1 and inducible Egr-1 (Figure 7B). There are differences in the intensity and location of the Sp1 and Egr-1 bands among the 10 different mutant DNAs. Sp1 and Egr-1 from nuclear extracts bound the wild type sequence with greater intensity than the mutant sequence; the relative intensity for mutants for Sp1 was 47 to 54 % of the wild type while the relative intensity for Egr-1 was 42 to 67% of the wild type. Recombinant Egr-1 (Figure 8) and Sp1 (data not shown) are both capable of binding all four oligonucleotides. Increasing 15 concentrations of Egr-1 in proportion to radiolabeled oligonucleotide produced multiple bands with differences in the pattern of binding between wild type and mutant oligonucleotides. The mutant consisting of a 6-bp addition produced three bands of greatest intensity, whereas the wild type and other mutants produced only two bands of lesser intensity. The wild type and 6-bp deletion of oligonucleotides 20 bound slightly more intensely than the 12-bp deletion oligonucleotide. Recombinant Sp1 produced only one intense band, and there were no obvious differences in binding intensity between the different oligonucleotides.

**EXAMPLE 2: Correlation of 5-Lipoxygenase Regulatory Polymorphisms with Responsiveness to Treatment with a 5-Lipoxygenase Inhibitor**

Patients with mild-to-moderate asthma who participated in one of two large clinical trials on the effectiveness of ZYFLO™ zileuton an asthma treatment 5 (Israel et al., *JAMA* 275:931, 1996, incorporated herein by reference) were contacted by letter and asked to provide genetic material for analysis.

Approximately 400 patients were contacted, 200 of whom had demonstrated a marked salutary response to ZYFLO™ zileuton and 200 of whom had not demonstrated such a response. Patients were asked to provide a buccal brush so 10 that samples including genetic material would be obtained for analysis.

Specifically, patients were given instructions to rinse their mouths out with water, to vigorously rub and rotate one brush on the inside surface of their right cheek for 30 second, and then to repeat the procedure with a second brush on their left cheek. Brushes were returned to us by mail. Responses were received from 109 15 individuals and genotyping was successful for 88 of them.

For each sample, we detected the number of Sp1 binding motifs in the 5-lipoxygenase transcription factor binding region. Since the wild-type 5-lipoxygenase allele has 5 Sp1 motifs, individuals homozygous for wild-type 5-lipoxygenase were designated "55". Similarly, individuals with one wild-type 20 allele and one allele containing only 4 Sp1 motifs were designated "54", etc.

The results are presented below in Table III:

TABLE III  
Genotype of ZYFLO™ Zileuton Study Patients

5

Genotype	Number of patients
55	43
45	31
44	10
35	2
34	1
56	1

10 The genotype information summarized above was correlated with the percent change in forced expiratory volume in the first second (FEV<sub>1</sub>), which was the primary outcome in the ZYFLO™ zileuton clinical trial. The data are presented below in Table IV.

15

20

25

TABLE IV  
Correlation of Genotype with FEV<sub>1</sub>

Number	Res/Non	%Predict ed FEV <sub>1</sub> @ baseline	%Change from baseline	Genotype
1	R	69.0	18.6	34
2	N	63.7	-1.5	35
3	R	36.5	31.3	35
4	N	76.1	-34.7	44
5	N	42.2	3.8	44
6	R	45.2	5.6	44
7	R	59.5	24.7	44
8	R	49.7	26.7	44
9	R	57.0	31.0	44

10	R	61.3	34.4	44	
11	R	43.5	34.5	44	
12	R	65.6	34.5	44	
13	R	51.5	63.5	44	
5	14	N	76.8	-45.2	45
15	15	N	77.5	-36.1	45
10	16	N	56.9	-33.0	45
19	17	N	77.5	-26.0	45
18	18	N	76.3	-19.9	45
10	19	N	78.8	-9.9	45
20	20	N	58.0	-9.5	45
21	21	N	59.4	-7.3	45
15	22	N	75.5	-6.7	45
23	23	N	74.3	-4.7	45
24	24	N	70.2	-2.5	45
25	25	N	77.1	-1.7	45
26	26	R	43.6	1.7	45
27	27	N	65.3	4.7	45
28	28	N	49.1	6.5	45
20	29	R	51.4	10.3	45
30	30	R	78.7	13.3	45
31	31	R	76.0	14.5	45
32	32	R	64.5	15.2	45
33	33	R	75.5	15.6	45
25	34	R	72.8	15.7	45
35	35	R	57.4	19.6	45
36	36	R	53.2	19.7	45
37	37	R	67.8	20.6	45

	38	R	54.2	24.7	45
	39	R	61.3	33.1	45
	40	R	41.9	35.1	45
	41	R	67.1	48.4	45
5	42	R	61.2	49.7	45
	43	R	43.8	65.6	45
	44	R	44.4	75.6	45
	45	N	42.0	-20.3	56
10		AVE	61.1	12.0	
		SD	12.7	27.2	
		SE	1.9	4.1	
	T-test	0.0449			
	46	N	81.7	-18.6	55
15	47	N	76.7	-17.9	55
	48	N	74.0	-17.2	55
	49	N	70.0	-14.7	55
	50	N	72.8	-12.4	55
	51	N	76.6	-11.2	55
20	52	N	67.4	-10.8	55
	53	N	78.8	-9.2	55
	54	N	71.2	-6.1	55
	55	N	76.4	-5.4	55
	56	N	67.7	-4.0	55
25	57	N	78.5	0.3	55
	58	N	71.2	0.3	55
	59	N	49.8	1.0	55
	60	N	67.4	1.7	55

	61	N	50.2	3.4	55
	62	N	62.8	5.3	55
	63	N	67.7	6.4	55
	64	R	47.3	8.4	55
5	65	R	41.9	12.5	55
	66	R	54.9	14.6	55
	67	R	71.1	25.4	55
	68	R	67.5	25.6	55
	69	R	65.6	29.8	55
10	70	R	65.5	30.1	55
	71	R	65.3	31.1	55
	72	R	54.1	32.7	55
	73	R	66.3	34.1	55
	74	R	61.5	35.4	55
15	75	R	61.3	36.2	55
	76	R	36.5	36.5	55
	77	R	67.7	37.0	55
	78	R	44.2	38.4	55
	79	R	45.0	40.1	55
20	80	R	42.2	44.7	55
	81	R	43.2	49.6	55
	82	R	53.9	56.6	55
	83	R	61.3	57.7	55
	84	R	52.6	62.5	55
25	85	R	55.4	64.2	55
	86	R	42.1	87.1	55
	87	R	36.6	91.3	55
	88	R	42.0	97.9	55

5

	AVE	60.6	22.6	
	SD	12.9	30.7	
	SE	2.0	4.6	

As can be seen, the FEV<sub>1</sub> outcome was significantly (P = 0.0449) greater in individuals harboring the wild-type genotype (55) than it was in the individuals harboring mutant genotypes. Specifically, individuals having the wild-type genotypes showed an average 22.6% increase in FEV<sub>1</sub> upon ZYFLO™ zileuton administration, whereas those with mutant genotypes showed an average 12% increase. These data suggest that individuals with mutant 5-lipoxygenase regulatory genotypes have reduced 5-lipoxygenase expression and therefore are benefitted less from pharmacologic down-regulation of the enzyme.

15 EXAMPLE 3: Large Scale Study to Identify Correlation Between 5-Lipoxygenase Regulatory Genotype with Response to Treatment with 5-Lipoxygenase Inhibitor

Over 500 patients were enrolled in a prospective study analyzing the response of their asthma to treatment with zileuton in a controlled-release formulation (ZYFLO™ zileuton). Each of these patients will be genotyped at the 5-LO gene, and 20 the clinical results will be stratified by genotype.

**Other Embodiments**

One of ordinary skill in the art will readily recognize that the foregoing has been merely a detailed description of certain preferred embodiments of the present invention. Various alterations and modifications of the procedures, techniques, 5 and compositions described above will be apparent to those in the art and are intended to be encompassed by the following claims.

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Grobholz, James
- (ii) TITLE OF INVENTION: 5-Lipoxygenase Gene Sequence  
Polymorphisms and Their Use in Classifying Patients
- (iii) NUMBER OF SEQUENCES: 1
- (iv) CORRESPONDENCE ADDRESS:
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- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
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- (viii) ATTORNEY/AGENT INFORMATION:
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2189 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: not relevant
- (ii) MOLECULE TYPE: DNA (genomic)
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: human 5-lipoxygenase gene (GenBank M 38191)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGATCCAGAA TAACCAAAAC AATATTGAAA AATAAAGAAC AGCGTTGGTG GATTAACATT  
 60  
 TTCCAATTTC AAAACTTACT ATAGCACTGC GGTAAATCAAG CAGTGTGGCA CTGTATAGCA  
 120  
 TGTACATTAC AGATCAGTGG ACTAGAAATCA ATGTCCAGAA ATAAACCGTT ATGTTTATAA  
 180  
 TGAATTACIT TTTAATAAGG TGTCAAGACA ACGCAATGGG AAAAGAATAA TGAATTCAAC  
 240  
 AAATGATGCA TGGACAACCG GACATGCACA TGCAACACAA TGAATTGAA TTCTTCTATC  
 300  
 GCTCCATGCA TAAAAACTAA CTCAAAATGG GTCACGGATG TAAATGAAAA GCTAAAACCA  
 360  
 TAATAATCCT AGAGGAAAAC CTAGGAGTAA ATCTTTAAGA TGTTATTGTA GGCAGTGGTT  
 420  
 TCTCAGATAG GACCCCAAAA TCACAAGCGA CAAAAAGAAA TTGGACTTAA AGTTAAATAC  
 480  
 TTTTGTGCTT CAAACATCAT CAAGAAAGTG AAAACACAAC CCGCAGAAGC AATAAAAATG  
 540  
 TCTGTAAGTC ATGTATCCGA TTAGAGACTT CTATCCAGGA TATATAAATA ATGCAATTCA  
 600  
 ATGATAAAAA AGATAAATAG CCCAGTTTC CAAAGAGTCAGCAGTAA TATACATCTC  
 660  
 TCCA AAAATA TACAGATATC CAACAAGCAT GTGAAAAGAT GTTCAAAGCC ATTTGCCAGG  
 720  
 TGCACAAACC CAAGACAGTA TGAGGAGATG CTACAGGGAC TCTGCTGCTT CACAGACATG  
 780  
 AAGCGTTGGT GAGAATGTAG GCAGCCGCCT TTGGGGACTT CACATCCCCG CCGCCCCACG  
 840  
 CACGGTGAGC TAGTGTAACTAGCCGA GATCAATACA CGCGACTGTG TGCCCGTCAG  
 900  
 ACCCTGCGCT GCCGGCGGGG CTGGGAGAGG CGGGCGCCAG GAGTGGGCGG GAACCTGGGG  
 960  
 GTCAGGCCCC AGCCGCGGGGA AGCGCCCCA GGAGCGCGCG AAACCTCTC CACACCCCTC  
 1020  
 CAGGCATTG CCCGCCGCGA TTCAGAGAGC CGACCCGTGA CCCCTGGCCT CCCCTAGACA  
 1080  
 GCCCGCGCATG TCCAGATGTG CCGTCCCGCC TGCCCTCCGC GACCACTGGC CATCTCTGGG  
 1140  
 CCTGGGCGCG GTTCTCGGCG CCCGGCCTGC CCCCGCCAGG AGCCGCAGGT CCAGCCAGTG  
 1200  
 AAGAAGCCCG CGCCTGAAGG AGCCTCTGTG CTCCAGAATC CATCCTCACT ATCAGCGCTG  
 1260

GGGTGGCCTC CTCCAGGAAG CCCTTCTGAT TCTCTCATGG GTCGCTCTTC CTCTGCAGAC  
1320  
TCCCGGAGCA CCCCCCTGCTC CAAGTACCGC AAGTGGCACT GAGAACCTGG GGAGAGCAGA  
1380  
GGCTGTGCCT AGATTTGTAG GGAGTCCCCG CAGCTCCACC CCAGGGCCTA CAGGAGCCTG  
1440  
GCCCTGGCG AAGCCGAGGC AGGCAGGCAG GCAAAGGGT GGAAGCAATT CAGGAGAGAA  
1500  
CGAGTGAACG AATGGATGAG GGGTGGCAGC CGAGGTTGCC CCAGTCCCCT GGCTGCAGGA  
1560  
ACAGACACCT CGCTGAGGAG AGACCCAGGA GCGAGGCCCC TGCCCCGCC GAGGCGAGGT  
1620  
CCCGCCCACT CGGCGCCGCG CGTGAAGAGT GGGAGAGAAG TACTGCGGGG GCGGGGGCGG  
1680  
GGCGGGGGC GGGGGCGGGG GCAGCCGGGA GCCTGGAGCC AGACCGGGGC GGGGCCGGGA  
1740  
CCGGGGCCAG GGACCAGTGG TGGGAGGAGG CTGCGGCCT AGATGCGGAC ACCTGGACCG  
1800  
CCGCGCCGAG GCTCCCGCG CTCGCTGCTC CCGCGGCCCG CGCCATGCC TCCTACACGG  
1860  
TCACCGTGGC CACTGGCAGC CAGTGGTTCG CCGGCACTGA CGACTACATC TACCTCAGCC  
1920  
TCGTGGCCTC GGCGGGCTGC AGCGAGAAGC ACCTGCTGGA CAAGCCCTTC TACAACGACT  
1980  
TCGAGCGTGG CGCGGTGAGC GCGGGCGGGG CACGGGTGGA GCGCGGGCTG AGGTGCGTCC  
2040  
GGGACCCGGT TTGGACGGCA GAGGCCTGGG CGGGGGCGCC GAGGGCCCGT CGGGGCGGCC  
2100  
CGGACAGGAC TGGGGGTGTC CAGGACCCCTG TCAGGGAGGG CAGAACTGCG GTGGGGCGTG  
2160  
CCCTGGCCTC CCAGTGGCCG GTGGGTACC  
2189

**Claims**

1. A method of classifying patients suffering from an inflammatory disease, the method comprising steps of identifying in DNA from at least one patient a sequence polymorphism, as compared with the normal 5-lipoxygenase gene (SEQ ID NO.:1), in a 5-lipoxygenase gene regulatory sequence; and classifying the patient based on the identified polymorphism.
2. The method of claim 1 wherein the inflammatory disease is selected from the group consisting of: asthma, ulcerative colitis, sinusitis, psoriasis, allergic and non-allergic rhinitis, lupus, and rheumatoid arthritis.
3. The method of claim 1 or claim 2 wherein the sequence polymorphism is selected from the group consisting of: addition of binding site for a transcriptional regulator expressed in white blood cells, and deletion of a binding site for a transcriptional regulator expressed in white blood cells.
4. The method of claim 1 or claim 2 wherein the sequence polymorphism is a nucleotide substitution selected from the group consisting of: a mutation that disrupts a binding site for a transcriptional regulator expressed in white blood cells, and a mutation that creates a binding site for a transcriptional regulator expressed in white blood cells.
5. The method of claim 3 or claim 4 wherein the transcriptional regulator is a transcriptional activator.
6. The method of claim 3 or claim 4 wherein the transcriptional regulator is a transcriptional repressor.

7. The method of claim 3 or claim 4 wherein the transcriptional regulator is selected from the group consisting of: Sp1, AP1, and NF $\kappa$ B, AP3, c-fos, CK-1, CK-2, c-myc, Egr-1, GATA, GC box, NF-AT, and TATA.

5 8. The method of claim 7 wherein the transcriptional regulator is selected from the group consisting of Sp1, AP1 and NF $\kappa$ B.

9. The method of claim 3 or claim 4 wherein the transcriptional regulator is Sp1 or Egr-1.

10

10. The method of claim 9 wherein the polymorphism comprises a deletion or disruption of at least one Sp1 or Egr-1 site.

11. The method of claim 10 wherein the at least one Sp1 site is selected from  
15 the group consisting of: the site at 1668-74 of SEQ ID NO.:1, the site at 1675-80  
of SEQ ID NO.: 1, 1681-86 of SEQ ID NO.: 1, 1687-92 of SEQ ID NO.: 1,  
1693-98 of SEQ ID NO.: 1, and combinations thereof.

12. The method of claim 10 wherein the polymorphism comprises a deletion of  
20 6 base pairs (GGGCGGG) at 1669 to 1674 of SEQ ID NO.:1.

13. The method of claim 10 wherein the polymorphism comprises a deletion of  
12 base pairs (GGGCGGGGGCGGG) at 1669-1680 of SEQ ID NO.:1.

25 14. The method of claim 9 wherein the polymorphism comprises addition of at  
least one Sp1 or Egr-1 site.

15. The method of claim 10 wherein the polymorphism comprises an addition  
of 6 base pairs (GGGCGGG) at 1688 of SEQ ID NO.:1.

30

16. A method of identifying an asthma patient who is a candidate for treatment with 5-lipoxygenase inhibitors, the method comprising steps of:
  - identifying a normal control individual who does not have asthma; and
  - identifying an asthma patient who expresses 5-lipoxygenase at a level
- 5 higher than that at which the normal control expresses 5-lipoxygenase, which asthma patient is therefore identified as a candidate for treatment with 5-lipoxygenase inhibitors.
17. A method of identifying an asthma patient who is not a candidate for treatment with 5-lipoxygenase inhibitors, the method comprising steps of:
  - identifying a normal control individual who has normal levels of 5-lipoxygenase gene expression; and
  - identifying an asthma patient who expresses 5-lipoxygenase at a level lower than that at which the normal control expresses 5-lipoxygenase, which asthma
- 10 patient is therefore identified as not being a candidate for treatment with 5-lipoxygenase inhibitors.
- 15
18. The method of claim 16 or claim 17 wherein the step of identifying an asthma patient comprises detecting levels of the 5-lipoxygenase protein in the asthma patient as compared with the normal control.
- 20
19. The method of claim 16 or claim 17 wherein the step of identifying an asthma patient comprises detecting levels of 5-lipoxygenase RNA in the asthma patient as compared with the normal control.
- 25
20. The method of claim 16 wherein the step of identifying an asthma patient comprises detecting an alteration in a 5-lipoxygenase gene regulatory sequence of the asthma patient as compared with the normal control.

21. The method of claim 20 wherein the step of identifying an asthma patient comprises detecting a disruption in a positive gene regulatory sequence of the asthma patient as compared with the normal control.
- 5 22. The method of claim 20 wherein the step of identifying an asthma patient comprises detecting an addition of a negative gene regulatory sequence in the asthma patient gene as compared with the normal control.
- 10 23. The method of claim 17 wherein the step of identifying an asthma patient comprises detecting an alteration in a 5-lipoxygenase gene regulatory sequence of the asthma patient as compared with the normal control.
- 15 24. The method of claim 23 wherein the step of identifying an asthma patient comprises detecting a disruption in a negative gene regulatory sequence of the asthma patient as compared with the normal control.
- 20 25. The method of claim 23 wherein the step of identifying an asthma patient comprises detecting an addition of a positive gene regulatory sequence in the asthma patient gene as compared with the normal control.
26. A method of identifying an individual who is susceptible to an inflammatory disorder, the method comprising steps of:
  - providing a nucleic acid sample from an individual;
  - detecting in the nucleic acid sample a 5-lipoxygenase gene polymorphism
- 25 that correlates with the inflammatory disorder with a P value less than or equal to 0.05, existence of the polymorphism being indicative of susceptibility to the inflammatory disorder.

27. The method of claim 26 wherein the inflammatory disorder is selected from the group consisting of asthma, ulcerative colitis, bronchitis, sinusitis, psoriasis, allergic, and non-allergic rhinitis, lupus, and rheumatoid arthritis.
- 5 28. A method of classifying an asthma patient as suffering from asthma, the method comprising steps of:
  - providing a nucleic acid sample from an asthmatic individual;
  - detecting in the nucleic acid sample a 5-lipoxygenase gene polymorphism that correlates with asthma with a P value less than or equal to 0.05, existence of
- 10 the polymorphism being indicative of asthma.
29. A method of treating inflammatory disorder patients, the method comprising:
  - identifying in genetic material of one or more inflammatory disorder
- 15 patients a 5-lipoxygenase gene polymorphism that correlates with increased responsiveness to a therapeutic as compared with responsiveness of an individual lacking the polymorphism; and
- administering the therapeutic to the patient.
- 20 30. The method of claim 29 wherein the therapeutic is selected from the group consisting of 5-lipoxygenase inhibitors and leukotriene receptor antagonists and the 5-lipoxygenase gene polymorphism comprises a 5-lipoxygenase gene mutation that reduces 5-lipoxygenase expression.
- 25 31. A method of determining the efficacy of treatment with 5-lipoxygenase inhibitors on a patient afflicted with an inflammatory disease, the method comprising steps of:
  - comparing the patient's 5-lipoxygenase gene sequence with the normal 5-lipoxygenase gene sequence (SEQ ID NO:1); and

determining whether or not there is any sequence polymorphism in the patient's 5-lipoxygenase gene sequence.

32. A method of determining the efficacy of treatment with 5-lipoxygenase inhibitors on a patient afflicted with asthma, the method comprising steps of: comparing the patient's 5-lipoxygenase gene sequence with the normal 5-lipoxygenase gene sequence (SEQ ID NO:1); and determining whether or not there is any sequence polymorphism in the patient's 5-lipoxygenase gene sequence.

10

33. The method of claim 31 or 32 further comprising one or more of the features of any one of method claims 1 to 25.

34. Use of one or more 5-lipoxygenase gene inhibitors for the manufacture of a medicament for the treatment of inflammatory disease in a patient having a polymorphism in his or her 5-lipoxygenase gene sequence.

35. Use of one or more leukotriene receptor antagonists for the manufacture of a medicament for the treatment of inflammatory disease in a patient having a polymorphism in his or her 5-lipoxygenase gene sequence.

36. Use of one or more 5-lipoxygenase activating protein inhibitors for the manufacture of a medicament for the treatment of inflammatory disease in a patient having a polymorphism in his or her 5-lipoxygenase gene sequence.

25

37. Use of any one of claims 34, 35, or 36 further including one or more of the features of any one of method claims 1 to 25.

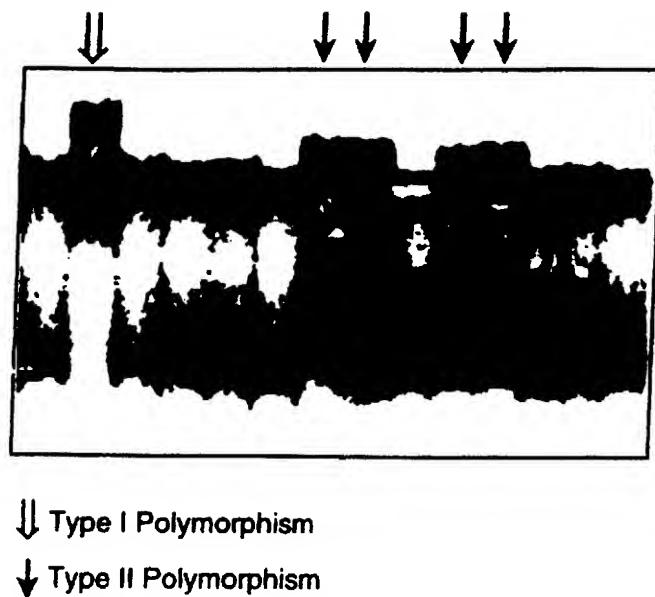


Figure 1.

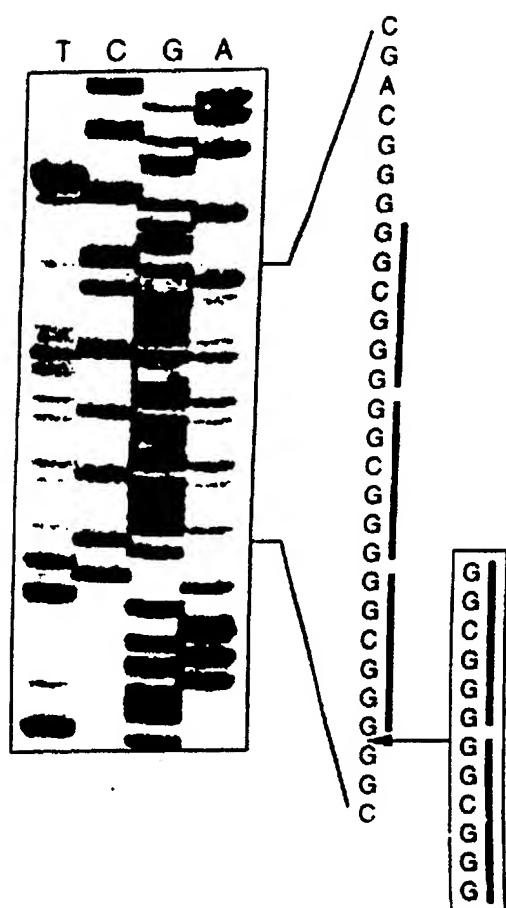


Figure 2.

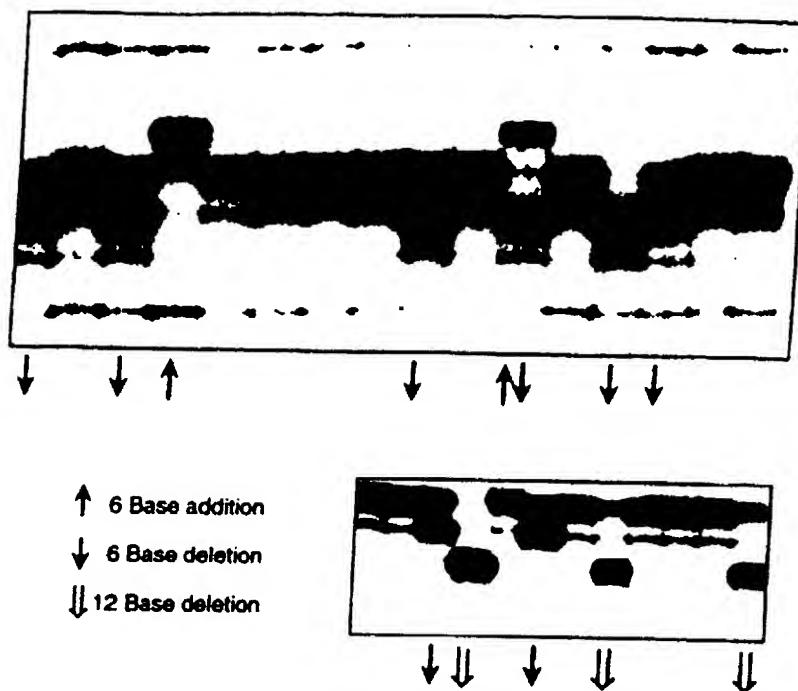
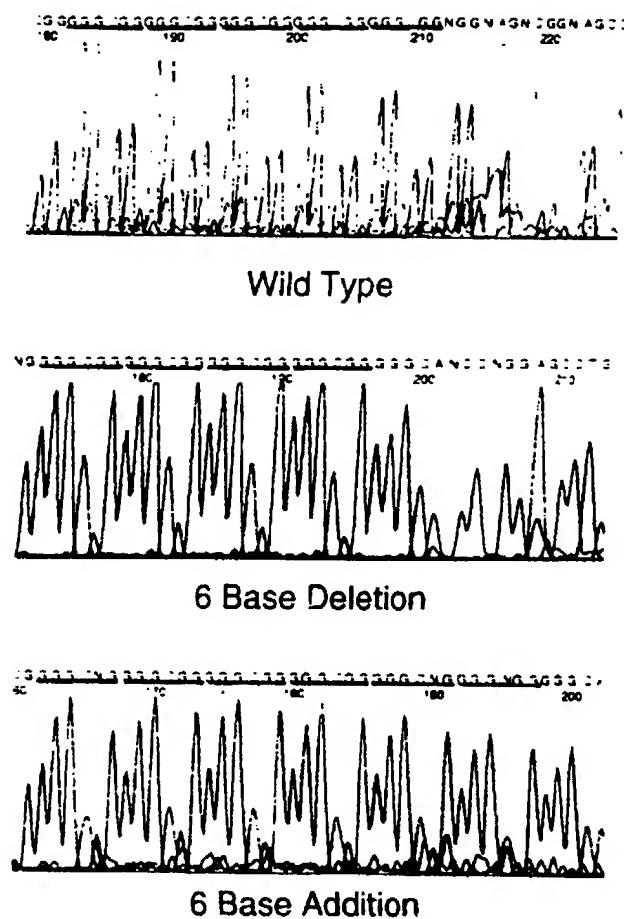


Figure 3.



**Figure 4.**

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	-180	-170	-160	-150	-140
#1	..... <u>GAGAAGTACT</u> GCGGGGGCGG	<u>GGGC</u> GGGG <u>GG</u> GG	<u>GGGG</u> GGGG <u>GG</u> GG	<u>GGGG</u> GGGG <u>GG</u> GG	CCGGAC...
#2	..... <u>GAGAAGTACT</u> GCGG.....	..... <u>GG</u> GC	<u>GGGG</u> GGGG <u>GG</u> GG	<u>GGGG</u> GGGG <u>GG</u> GG	CCGGAC...
#3	..... <u>GAGAAGTACT</u> GCGG.....	<u>GGGC</u> GGGG <u>GG</u> GC	<u>GGGG</u> GGGG <u>GG</u> GG	<u>GGGG</u> GGGG <u>GG</u> GG	CCGGAC...
#4	..... <u>TNCTNCCGGG</u> GCGGGGGCGG	<u>GGGC</u> GGGG <u>GG</u> GC	<u>GGGG</u> GGGG <u>GG</u> GG	<u>GGGG</u> GGGG <u>GG</u> GG	CCGGAC...

SP-1 binding motifs (GGGGCGG) are shown underlined alternating with shaded.

- #1 Normal sequence of the tandem SP1 binding motifs in the transcription factor binding region of the 5-lipoxygenase gene. Sequence numbering is from the GENBANK accession #M38191. SEQ HUMAN 5-LIPOXYGENASE GENE EXON #1-1/91
- #2 Sequence with the 12 base pair deletion
- #3 Sequence with the 6 base pair deletion
- #4 Sequence with the 6 base pair addition; note that sequence 5' of the insertion is poorly resolved.

FIG. 5

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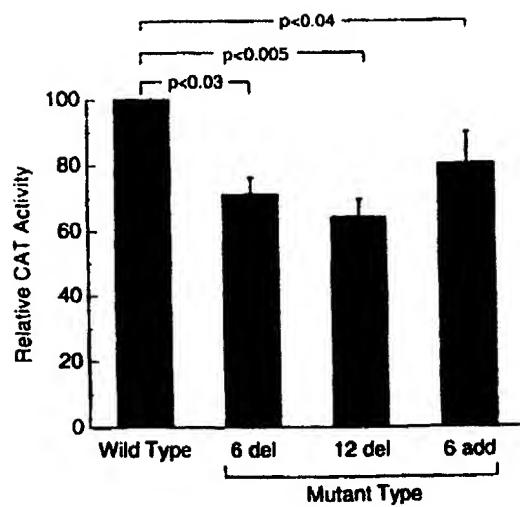


FIGURE 6

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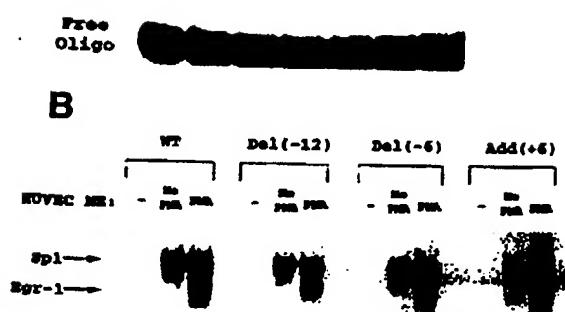
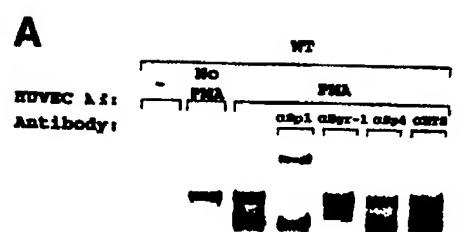


FIGURE 7

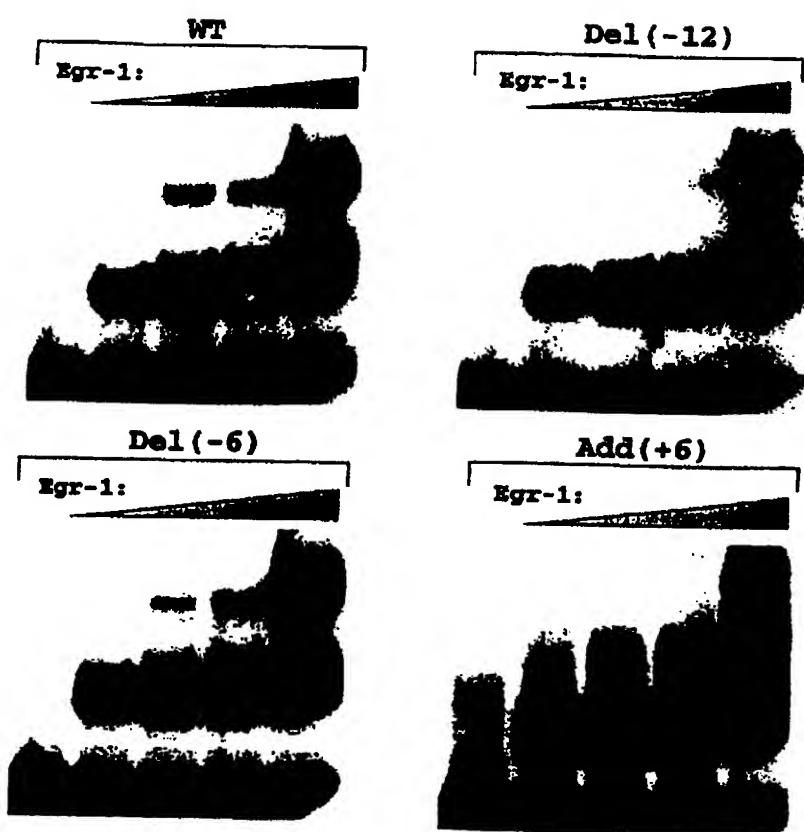


FIGURE 8